IDENTIFICATION AND PURIFICATION OF A NICOTINAMIDE ADENINE DINUCLEOTIDE-DEPENDENT SECONDARY ALCOHOL DEHYDROGENASE FROM C_1-UTILIZING MICROBES

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1. Introduction

Phenazine methosulfate (PMS)-dependent methanol dehydrogenase has been reported from many methylotrophic bacteria [1–4]. This enzyme oxidizes primary alcohols from C_1–C_{10} but does not oxidize secondary alcohols. Nicotinamide adenine dinucleotide (NAD)-dependent alcohol dehydrogenases have been reported from liver and yeast [5]. These alcohol dehydrogenases oxidize primary alcohols and acetaldehyde but have no activity on methanol. In addition, the alcohol dehydrogenases from yeast and liver also oxidize some secondary alcohols at a very low rate (<1% of their ethanol activity). NAD(P)-dependent alcohol dehydrogenases were also reported in *Pseudomonas* [6,7], *Escherichia coli* [8] and *Leuconostoc* [9]. However, these enzymes were active only on long-chain primary alcohols or hydroxy fatty acids [7].

To our knowledge, no secondary alcohol-specific alcohol dehydrogenase (SADH) has been reported. We have recently identified an NAD-linked, secondary alcohol-specific, alcohol dehydrogenase in cell-free extracts of various gaseous hydrocarbon-utilizing microbes. This enzyme is also found in cells grown on methanol. It specifically and stoichiometrically oxidizes secondary alcohols to their corresponding methyl ketones. This enzyme has been purified 2600-fold and shows a single protein band on acrylamide gel electrophoresis.

2. Materials and methods

2.1. *Bacterial strains*

Methylosinus trichosporium OB3b was kindly provided by R. Whittenburg (University of Warwick, Coventry). *Methylococcus capsulatus* CRL M1 was isolated from the lake water of Warinaco Park, Linden, NJ. Other microbes were obtained from the American Type Culture Collection. The organisms were maintained at 30°C on mineral salts [10] agar plates in a dessicator with either methane or methanol.

2.2. *Chemicals*

Diethyl aminoethyl (DEAE)-cellulose and NAD(P) (both oxidized and reduced forms) were purchased from Sigma Chemical Co. (St Louis, MO). All alcohols were obtained from Matheson Coleman and Bell Manufacturing Co. (Norwood, OH). Bio-Gel agarose A-1.5 and Affi-Gel Blue (50–100) were obtained from Bio-Rad Lab.

2.3. *Enzyme assay*

NAD-linked alcohol dehydrogenase activity was measured with a fluorescence spectrophotometer (Perkin Elmer, Mode MPF 44A) by following the formation of reduced NAD (EX 340 nm, EM 460 nm). The assay system (3 ml) contained: sodium phosphate buffer (pH 7.0) 150 μmol; NAD 1.0 μmol; secondary alcohol 20 μmol; and a given amount of enzyme preparation. One unit of enzyme activity represents the reduction of one nmol NAD per minute. Protein concentrations were determined by the Lowry method [11].

2.4. *Identification of product and stoichiometry*

The methyl ketones were identified by retention time comparisons and co-chromatography with authentic standards using flame ionization gas chro-

matography. A stainless-steel column (20 ft × 1/8 in packed with 10% Carbowax 20M on 80/100 Chromsorb W, Supelco, Inc., Bellefonte, PA) was maintained isothermally at 110°C. Quantity of the methyl ketones was calculated from the peak area with a standard curve which had been constructed with authentic standards. For reaction stoichiometry, secondary alcohol dehydrogenase (SADH) (10 μg) was incubated with the standard enzyme assay system. After 10 min reaction, the amount of 2-butanol and the amount of 2-butanone produced were assayed by gas chromatography. The amount of NADH formed was measured by fluorescence spectrophotometer. A 0.65 μmol reduction of NAD corresponded stoichiometrically with the consumption of 0.65 μmol 2-butanol and the formation of 0.65 μmol 2-butanone. No consumption of oxygen was observed (determined with a Clark oxygen electrode, Yellow Springs Instrument Co., OH).

2.5. *Acrylamide gel electrophoresis*

Acrylamide gel electrophoresis was conducted in 7.5% gel and stained with Coomassie brilliant blue [12]. Sodium dodecyl sulfate–gel electrophoresis (10% gel) was according to Weber and Osborn [13].

2.6. *Enzyme purification*

SADH from an obligate methanol utilizer, *Pseudomonas* sp. ATCC 21439, has been purified. The cells suspended in 300 ml 0.05 M sodium phosphate buffer (pH 7.0) with 0.5 mM dithiothreitol (buffer A) were disrupted sonically (5 × 1 min). The crude extract was separated by centrifugation. The crude extract was heat-treated in a 50°C water bath for 10 min. The resulting precipitate was removed by centrifugation. To the supernatant solution, 25 ml protamine sulfate solution (2% solution in 0.1 M Tris base) was added dropwise with continuous stirring. After standing for 30 min, the extract was centrifuged. The supernatant solution was fractioned with solid ammonium sulfate. Material precipitating at 30–60% ammonium sulfate saturation was collected and was dialyzed overnight against buffer A. The dialyzed material was applied to a DEAE-cellulose column (3 × 35 cm) that had been equilibrated with buffer A. The secondary alcohol dehydrogenase activity was eluted in the void volume. This DEAE-cellulose eluate was concentrated by ammonium sulfate fractionation. Material precipitating at 30–50% ammonium sulfate saturation was collected by centrifugation and dialyzed overnight against buffer A. This fraction was further washed and filtered through an Amicon unit with XM 50 membrane. The concentrated fraction (6 ml) inside the Amicon unit was applied to an Affi-Gel Blue column (0.8 × 18 cm) which had been equilibrated with buffer A for affinity chromatography. The column was washed overnight with buffer A (0.18 ml/min) and then was eluted with buffer A containing 5 mM NAD. Each 1 ml fraction was collected. SADH activity was located in tube no. 8–12. A summary of the purification steps is given in table 1.

Table 1
Purification of secondary alcohol dehydrogenase from *Pseudomonas* sp. ATCC 21439

Procedures	Volume (ml)	Protein (mg)	Spec. act. (units/mg protein)	Total units	Yield (%)
Crude extract	250	2698	25	67 450	100
Heat treatment	245	949	67.5	64 080	95
Protamine sulfate	260	526	103.8	54 640	81
$(NH_4)_2SO_4$ (30–60% sat.)	30	232	200	46 450	69
DEAE-cellulose column	150	42.2	875	37 160	55
Amicon filtration (XM-50)	6	22.0	1500	33 050	49
Affi-Gel Blue column	5	0.34	65 600	22 300	33

3. Results and discussion

3.1. *Methyl ketone formation*

Methane-grown *Pseudomonas methanica* was reported [14] to co-oxidize propane and butane to their corresponding methyl ketones. They stated that resting cell-suspensions of methane-grown cells, however, did not oxidize propane or butane. Propane-grown *Myobacterium smegmatis* 422 was reported [15] to oxidize *n*-alkanes to their corresponding methyl ketones. We have found resting cell-suspension of methane- or propane-grown cells do oxidize propane and butane. In addition, these resting cell suspensions also oxidized secondary alcohols to their corresponding methyl ketones. Furthermore, the resting cell suspensions of primary alcohol-grown cells also converted secondary alcohols to their corresponding methyl ketones. The conversion of 2-butanol to 2-butanone by cell-free crude extracts of various distinct types of methylotrops grown on either methane or methanol is listed in table 2.

3.2. *Cofactor requirement*

NAD was found to be a requirement for the oxidation of secondary alcohols in the cell-free system. Other cofactors tested (including PMS, GSH, FAD, potassium ferricyanide, dichlorophenol indophenol and NADP) were not effective.

3.3. *Properties of SADH*

The molecular weight of the pure SADH as estimated by a Bio-Gel agarose A-1.5 column is 95 000. Acrylamide gel electrophoresis of the purified SADH fraction from the affinity chromatography showed a single protein band (fig.1). SDS–gel electrophoresis (10% gel) of the purified SADH fraction showed two identical subunits of 48 000 mol. wt. The K_m values for 2-butanol and NAD are 0.25 mM and 0.011 mM, respectively. The pH optimum for SADH activity was ~8–9.

3.4. *Substrate specificity*

SADH oxidizes secondary alcohols with the following relative % rates: 2-propanol (85%); 2-butanol (100%); 2-pentanol (5%); 2-hexanol (2%); acetaldehyde (4%); propanol (2%); cyclohexanol (4%); butane 1,3-diol (2%); butane 2,3-diol (2.5%). The following compounds tested were not oxidized by SADH: 2-heptanol to 2-decanol; formaldehyde; butanal to decanal benzaldehyde; methanol to *n*-decanol; isobutanol; phenol; butane 1,2-diol; succinic acid. It seems that a hydrophobic carbon moiety adjacent to

Table 2
Conversion of 2-butanol to 2-butanone by soluble crude extracts of methylotrophs

Microbes	Growth substrate	Conversion (units/mg prot.)
Obligate methylotrophs		
Type II membrane structure		
Methylosinus trichosporium OB3b	CH_4	4.5
Methylosinus trichosporium OB3b	CH_3OH	2.4
Type I membrane structure		
Methylococcus capsulatus CRL M1	CH_4	3.2
Methylococcus capsulatus CRL M1	CH_3OH	2.0
Facultative methylotrophs		
Methylobacterium organophilum CRL 26	CH_4	1.8
Methylobacterium organophilum CRL 26	CH_3OH	2.5
Others		
Pseudomonas sp. ATCC 21 439	CH_3OH	25
Hansenula polymorpha ATCC 26 012	CH_3OH	23

Cells were disrupted sonically as described in the text. The supernatant of 10 000 × *g* centrifugation was used for the enzyme assay

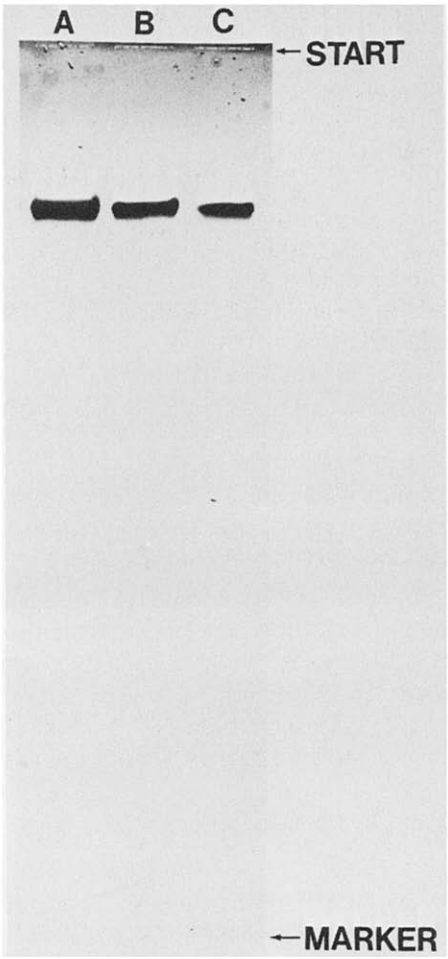

Fig.1. Gel electrophoretic pattern of secondary alcohol dehydrogenase from *Pseudomonas* sp. ATCC 21439. Enzyme 40 μg (A), 20 μg (B) and 10 μg (C) were applied to each slot. Protein migrated to the anode (bottom) at a constant 300 mV for 2 h in 0.05 M Tris–glycine buffer (pH 9.0).

the secondary alcohol is required for the enzyme activity.

3.5. *Inhibition studies*

The SADH activity was inhibited by metal-chelating agents in the following order (% inhibition): 1,10-phenanthroline (95%); α,α-bipyridyl (70%); EDTA (63%); sodium azide (10%). This suggests possible metal involvement. However, the activity was not inhibited by sodium cyanide or thiourea. The enzyme activity was also inhibited by strong thio inhibitors such as *p*-hydroxy mercuribenzoate (100%) and 5,5′-dithiobis(2-nitrobenzoic acid) and was not inhibited by less potent thio inhibitors such as iodoacetic acid or *N*-ethylmaleimide.

We have also identified SADH activity in gaseous hydrocarbon utilizers other than methylotrophs (C.T.H. et al. in preparation). The physiological significance of this SADH in methyltrophs as well as other gaseous hydrocarbon utilizers is not known. However, possessing this enzyme is of great advantage to the organism as its growth yield, when growing on gaseous alkanes as the sole source of carbon and energy, could be exclusively NAD(P)H-dependent [16,17]. Secondary alcohols are intermediates in the oxidation of *n*-alkanes by either *Pseudomonas* or *Mycobacterium* [15]. The methane monooxygenase from *Methylococcus capsulatus* (Bath) and others also oxidizes *n*-alkanes to both primary and secondary alcohols [18,19]. Therefore, the presence of a secondary alcohol dehydrogenase in these gaseous hydrocarbon utilizers seems not a complete surprise. The fact that SADH is also present in the methanol-grown cells indicates that the enzyme is not induced by *n*-alkanes. The extent of resemblance between this SADH and the well-characterized alcohol dehydrogenases from liver and yeast as to their catalytic sites and enzyme protein conformations requires further investigation.

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